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The goal of this project has been to optimize the Threshold DNA probe assay as a method for quantitating specific sequences after amplification by the polymerase chain reaction. Increasing the probe concentration 5-fold in the standard scheme B assay results in more rapid annealing of the probes to the target DNA, decreasing the overall assay time to less than 1 hour. A second assay format was developed in which the capture reagent and enzyme conjugate are added simultaneously. This scheme A format gives comparable results to those obtained using scheme B, and shortens the assay time to less than 30 minutes. Studies were also initiated to increase the detection sensitivity of the Threshold DNA probe assay by introducing multiple labels into the probe sequence by PCR. Data obtained after the reporting period officially ended indicate that 10 genomic targets can be detected after 25 cycles of PCR by using longer probes containing multiple fluorescein labels.

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RAPID DETECTION OF INFECTIOUS AGENTS WITH A BIOSENSOR-BASED NUCLEIC ACID HYBRIDIZATION ASSAY

Final Report
Contract # N00014-91-C-0279
Reporting Period 9/30/91-9/30/92

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ABSTRACT

The goal of this project has been to optimize the Threshold DNA probe assay as a method for quantitating specific sequences after amplification by the polymerase chain reaction. Increasing the probe concentration 5-fold in the standard scheme B assay results in more rapid annealing of the probes to the target DNA, decreasing the overall assay time to less than 1 hour. A second assay format was developed in which the capture reagent and enzyme conjugate are added simultaneously. This scheme A format gives comparable results to those obtained using scheme B, and shortens the assay time to less than 30 min. Studies were also initiated to increase the detection sensitivity of the Threshold DNA probe assay by introducing multiple labels into the probe sequence by PCR. Data obtained after the reporting period officially ended indicate that 10 genomic targets can be detected after 25 cycles of PCR by using longer probes containing multiple fluorescein labels.

MATERIALS AND METHODS

Biotinylated and single fluoresceinated probes were synthesized by Applied Biosystems Inc. The sequences of the pGEM-specific probes are given in reference 1. The anthrax-specific probes were obtained from NMRI. Dual fluoresceinated probes were synthesized by Clontech. The standard scheme B probe assay protocol (prior to the modifications described in this report) is as follows: The target DNA to be detected is diluted into a final volume of 25 µl of hybridization buffer (30 mM sodium phosphate, 450 mM NaCl, 3 mM EDTA, 0.25% Triton X-100, pH 7.4). Probes are diluted in hybridization buffer so that 25 µl contains 1.8×10^{11} molecules of each probe. 25 µl of probe solution and 25 µl of target solution are mixed in PCR tubes. In a thermal cycler, the reactions are denatured at 100°C then annealed at 53°C. The tubes are then transferred to ice. 100 µl of capture reagent containing 2 µg of streptavidin in assay buffer (10 mM sodium phosphate, 1 mM EDTA, 150 mM NaCl, 0.05% Tween 20, 0.1% BSA, pH 7.0) is added to each tube and the samples are transferred to the filtration unit wells of the Threshold instrument. Vacuum is applied at low setting (~100 µl/min) and then 1 ml of wash buffer (10 mM sodium phosphate, 1 mM EDTA, 150 mM NaCl, 0.05% Tween 20, pH6.5) is filtered through each well under high vacuum (~500 µl/min). The vacuum is turned off and 200 µl of α-fluorescein-urease conjugate in assay buffer is added to each well and incubated for 30 min. The remaining conjugate is filtered under low vacuum, 1 ml of wash

buffer is filtered through under high vacuum, and the sticks are then stored in wash buffer until read.

RESULTS

Optimization of scheme B

The standard scheme B protocol involves a 15 minute denaturation of the target DNA to be detected and a 15 minute annealing step with the biotinylated and fluoresceinated probes (1). The annealing time could be reduced if the concentration of probes was increased. In order to determine if increasing the probe concentration would increase the background signal, various probe concentrations were tested in hybridization buffer (no target DNA present) in the scheme B format. Increasing the concentration of both probes up to 10-fold did not significantly increase the background signal (data not shown).

The effect of increasing the probe concentration on the kinetics of hybridization to $2x10^9$ targets was examined. Using the standard probe concentration ($1.8x10^{11}$ molecules of each probe per assay), at least 2-5 minutes of annealing time is required to produce maximum Threshold signal (figure 1). However, by increasing the probe concentration 5 or 10-fold, maximum Threshold signal is obtained when the samples are transferred directly to ice from the denaturation temperature, essentially reducing the annealing time to 0 minutes (figure 1). The standard scheme B assay now uses $9x10^{11}$ molecules of each probe per assay (5X the original concentration).

The effect of the length of time the α-fluorescein-urease enzyme conjugate incubates with the captured probe-target complex on the Threshold stick on the signal was evaluated using the new standard amount of probes. It was found that the to obtain maximum signal at the high end of the standard curve, the conjugate must incubate at least 20-30 minutes on the membrane (figure 2). At the low end of the standard curve, however, it appears that a 10 minute incubation is sufficient. The final optimized scheme B protocol is given in appendix I.

Development and optimization of scheme A

A significant reduction in the total time required for the Threshold DNA probe assay would be achieved if the 30 minute incubation time with the enzyme conjugate could be eliminated. It was postulated that if the enzyme conjugate

were added to the probe-target complex in solution, rather than after the complex had been filtered through the Threshold membrane, the fluoresceins on the probe would be more accessible and the conjugate would bind much faster. A new assay format was devised in which the enzyme conjugate is added to the samples at the same time as the capture reagent.

However, in this format any fluorescein probe not annealed to target will still be present when the conjugate is added and will compete with the bound probe. An experiment was therefore done in which the probe concentration, the amount of enzyme conjugate added, and the type of conjugate used were varied in order to determine which combination would yield the highest signal in the scheme A format. Conjugates were made with polyclonal α-fluorescein or monoclonal α-fluorescein. The monoclonal α-fluorescein antibody was obtained from Scripps Clinic for government investigational purposes. As shown in figure 3, the highest signal for $2x10^9$ target molecules in scheme A was achieved with 200µl of the monoclonal α-fluorescein-urease conjugate in combination with $9x10^{10}$ molecules of each probe (0.5X).

The kinetics of probe hybridization with $9x10^{10}$ molecules of each probe in the scheme A format was examined with $8x10^9$ target molecules. It was found that a 5 minute annealing time is necessary in the scheme A format to get maximum signal (figure 4). The effect of the length of time the enzyme conjugate is incubated with the probe-target mixture prior to filtering was also examined. As shown in figure 5, the conjugate incubation time in scheme A does not have a significant effect on the signal in the probe assay at the low end of the standard curve. The optimized scheme A protocol is given in appendix II.

The optimized scheme B and scheme A protocols were run side by side and as shown in figure 6, the two formats give comparable results, although the signals in the scheme B format are slightly higher at the high end of the standard curve. A possible explanation for this observation is that, at high target numbers in the scheme A protocol, the second strand of the target may displace the bound probes during the 5 min annealing period, resulting in a lower signal. Since there is no annealing period in the scheme B protocol, the reduction in signal due to strand displacement is not observed.

Construction of model target plasmid pKHant

The initial studies performed with the anthrax-specific probes used the 622 bp PCR product which had been gel purified and quantitated by Tim Reif and

Suresh Pillai at NMRI. In order to ensure a steady supply of easily quantitated target DNA for the experiments discussed in this report, a portion of the 622 bp anthrax PCR product was subcloned into the vector pGEM-3Z (available from Promega). The gel purified fragment was digested with *Rsa* I, which cuts within the leftward PCR primer BACA1F1, and *Bam* HI, which cuts at the 3' end of the fluorescein probe sequence. The vector pGEM-3Z was digested with *Bam* HI and *Hinc* II, both of which cut within the polylinker sequence. (The enzymes *Rsa* I and *Hinc* II leave compatible ends which can ligate to each other). The vector was also treated with calf intestinal alkaline phosphatase to prevent re-ligation of vector without insert. The prepared vector and insert were ligated and transformed into *E. coli* strain DH5α. A restriction map of the resulting plasmid, pKHant, is shown in figure 7. A large scale preparation of this plasmid was made and the DNA was quantitated by OD_{260}. The quantitated plasmid was digested with *Bam* HI and *Sph* I to release the insert and this digest was then used as the target in all future assays using the anthrax-specific probes.

Dual fluoresceinated probes

In the standard Threshold DNA probe assay, one of the probes contains a single fluorescein capable of binding a single α-fluorescein-urease conjugate. An obvious way to increase the sensitivity of the assay, then, would be to increase the number of fluoresceins on the probe which would result in more conjugate binding to the probe-target complex. To test this hypothesis, the 30-mer probe for the anthrax target (PACAFL2) was re-synthesized with 2 fluoresceins, one at the 5' end and one at the 3' end. Using this dual fluoresceinated probe to detect the anthrax target would be expected to yield twice the signal obtained with the single labeled probe. However, when the single and double labeled probes were used to generate standard curves in a side by side comparison, it was found that there was no enhancement of signal with the dual fluoresceinated probe (figure 8).

Experiments were performed to confirm the presence of two fluoresceins on the newly synthesized probe and to assess the availability of these fluoresceins to binding by α-fluorescein-biotin or α-fluorescein-urease. These studies indicated that there are two fluoresceins present on each probe and that they are both available for binding, but that there is significant steric hindrance in binding two α-fluorescein-urease conjugates only 30 bases apart. (This was found to be true both when the probe was free and when annealed to target).

Since there are 10 base pairs per turn of double-stranded DNA it would be expected that the 2 fluoresceins on the probe would be located on the same side of the helix. Perhaps if the fluoresceins were located on opposite sides of the double helix when annealed to target, the steric hindrance in binding two conjugate molecules would be eliminated. To test this, a 35-mer probe with a fluorescein at the 5' end and at the 3' end was synthesized. This dual fluoresceinated probe, however, was also found not to enhance the signal in the assay (data not shown).

Reduction of background signal

Several experiments were performed to try to lower the background signal in the probe assay by reducing the non-specific binding of the enzyme conjugate to the Threshold membrane. Threshold sticks were pretreated by filtering through 0.5 ml of different concentrations of various blocking agents before filtering the sample. These included non-fat dry milk, casein, and Denhardt's solution. Although pre-treatment with these agents did reduce the background signal, they also reduced the specific signal, and were not investigated further. However, simply filtering 0.5 ml of wash buffer through the sticks (vacuum on high setting) before applying the sample was found to decrease the background signal by 15-20% without affecting the specific signal (data not shown).

Introduction of multiple fluoresceins via PCR

Recently, kits have become available from Amersham which permit the incorporation of fluorescein-dUTP into DNA during random prime labeling or PCR. Experiments were designed to determine if the sensitivity of the Threshold DNA probe assay could be increased by incorporating multiple fluoresceins into longer, double-stranded probes via PCR or by incorporating multiple fluoresceins directly into the anthrax target during the standard 25 cycles of PCR.

PCR reactions were set up using the 5'-fluoresceinated probe (PACAFL2) and oligo BACA6R1 as the primers with varying ratios of Fl-dUTP:dTTP. As shown in figure 9A, lane 2, these primers amplify an approximately 114 bp fragment from the anthrax target DNA. Amplification is also quite efficient when a ratio of 75% Fl-dUTP to 25% dTTP is used (figure 9A, lane 3). The PCR product is larger in this reaction, presumably due to the incorporation of multiple fluoresceins. However, using 100% Fl-dUTP in PCR yields almost no product (figure 9A, lane 4).

The PCR reaction containing 75% Fl-dUTP was then used as the fluoresceinated probe in the Threshold probe assay. Since the amount of fluoresceinated probe produced by PCR was not quantitated, an arbitrary amount of the PCR reaction was added, along with the standared amount of the 30-mer biotinylated probe, to the samples. It was found that the signals obtained with the PCR generated probes were higher if a protocol which is a combination of schemes A and B is used. The scheme A/B protocol is given in appendix III. Results of a scheme A/B assay using the 114 bp Fl-PCR reaction compared to the single fluoresceinated 30-mer are shown in figure 9B. Use of the Fl-PCR reaction as the fluorescein probe enhances the signal approximately 2-fold.

An alternative to generating multiply fluoresceinated probes is to incorporate Fl-dUTP directly into the genomic PCR reactions. The Threshold DNA probe assay would then be run by adding just the biotinylated probe. PCR reactions with varying numbers of anthrax genomic targets were performed in the presence and absence of Fl-dUTP (50%) for 25 cycles. Aliquots of the reactions were analyzed on a 1.2% agarose gel stained with ethidium bromide (figure 10A). The reactions containing 10^4 starting genomic targets clearly show a visible product on the gel (figure 10A, lanes 3 and 8). However, the reaction containing Fl-dUTP gives a much fainter band which indicates that the PCR amplification is less efficient when 50% of the dTTP is replaced with Fl-dUTP. The reaction containing 10^3 starting targets but no Fl-dUTP shows a very faint band. All other reactions show no discernible product band.

A scheme B assay was run on the Fl-PCR reactions using only the biotinylated 30-mer probe and the results are shown in figure 10B. The signals obtained for the genomic Fl-PCR reactions are essentially the same as those obtained for the control reactions using both the fluoresceinated and biotinylated 30-mer probes. However, since the gel analysis demonstrated that the Fl-PCR reactions amplified much less efficiently, there is less target in these reactions than in the controls. Also, the Fl-PCR reaction containing 10^3 starting targets shows no band on a gel while the control reaction shows a faint band, yet the Fl-PCR reaction gives a signal of 807.8 uv/sec in the probe assay while the control reaction gives a signal of 552.1 uv/sec.

Experiments were performed to try to improve the efficiency of PCR in the presence of Fl-dUTP, as has been done previously for incorporation of digoxigenin-dUTP (2, 3). Reactions were set up with 10^4 anthrax genomic targets and varying ratios of dTTP to Fl-dUTP. After 25 cycles of amplification,

aliquots were analyzed on an agarose gel (figure 11A). It was found that a 1:1 ratio of Fl-dUTP to dTTP was optimal for PCR (lane 6), but the control reaction containing 100% dTTP still amplified more efficiently (lane 3). However, when the probe assay was run on these reactions, the reaction containing 50% Fl-dUTP gave a signal 3 times higher than that of the control reaction (13139.0 uv/sec compared to 4423.1 uv/sec; figure 11B) even though the gel analysis indicates that the control reaction contains more target. It was also observed that the intensity of the product band decreased in reactions with less than 50% Fl-dUTP (figure 11, lanes 6-8). A possible explanation is that the intensity of the product bands on a gel is a combination of ethidium bromide fluorescence and that of the incorporated fluorescein. Therefore, there may actually be more DNA in lane 8 but the combined fluorescence of the DNA and incorporated fluorescein in lane 6 makes it appear that there is more DNA present.

After many experiments, it was concluded that introduction of multiple fluoresceins directly into the genomic target during PCR is too inconsistent to be used as a routine method for increasing the sensitivity of the Threshold DNA probe assay. Experiments performed after the conclusion of the contract between NMRI and MDC indicate that a much greater and reproducible enhancement of sensitivity is achieved by generating probes containing multiple fluoresceins via PCR. These experiments will be described in a separate report.

CONCLUSIONS

The standard Threshold DNA probe assay has been optimized and total assay time reduced to less than 1 hour. A second assay format has been developed which reduces the total assay time to less than 30 minutes with no loss in sensitivity. Initial studies indicate that assay sensitivity can be greatly increased by incorporation of multiple fluoresceins into probes generated by PCR.

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2. Stürzl, M., Oskoui, K.B. & Roth, W.K. (1992). 'Run-off' polymerization with digoxigenin labelled nucleotides creates highly sensitive and strand specific hybridization probes: synthesis and application. *Molecular and Cellular Probes* **6**, 107-114.

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LEGENDS TO FIGURES

Figure 1. A. $1.8x10^{11}$ (1X), $9x10^{11}$ (5X), or $1.8x10^{12}$ (10X) molecules of each probe for the anthrax model system were mixed with $2x10^9$ molecules of the 622 bp anthrax PCR product (gel purified) in a total volume of 50 µl of hybridization buffer. Reactions were then denatured at 100°C for 5 min and then annealed at 53°C for various times (0-15 min) before transferring to ice. Samples were then processed as in scheme B.

Figure 2. Four sets of reactions were set up using the pGEM/HhaI target and probes. After the samples were filtered onto the Threshold sticks, conjugate was added to one of the four sticks. After 10 min conjugate was added to a second stick and 10 min later to a third. After a total of 30 min conjugate was added to the fourth stick and all sticks were filtered on low vacuum simultaneously and then washed and read as usual.

Figure 3. Reactions contained $2x10^9$ pGEM/HhaI targets and various concentrations of probes (0.1X-5X). After denaturation, annealing, and addition of capture reagent, different amounts of ILA conjugate or monoclonal conjugate #143 were added. The no target negative controls were set up only with 0.5X and 1X concentration of probes.

Figure 4. Reactions contained $8x10^9$ pGEM/HhaI targets and $9x10^{10}$ molecules of each probe (0.5X). Reactions were denatured at 100°C for 5 min and then annealed at 53°C for 0, 1, 2, 5, 10, or 15 min before transferring to ice. Capture reagent and either 500 µl of ILA conjugate or 200 µl of monoclonal conjugate were added. No target negative control reactions were annealed for 5 min.

Figure 5. Four sets of reactions were set up with the pGEM target and probes. After denaturation and annealing, capture reagent and monoclonal conjugate were added to one set of reactions. After 10 min capture reagent and conjugate were added to the second set of reactions and 10 min later to the third. After a total of 30 min capture reagent and conjugate were added to the final set of reactions. All sets were then processed simultaneously.

Figure 6. Standard curves were generated using the optimized scheme A and scheme B protocols (appendices I and II) side by side with the pGEM model system.

Figure 7. The plasmid pKHant contains a 585 bp portion of the 622 bp anthrax PCR product cloned into the vector pGEM-3Z. The location of the ampicillin resistance gene (Amp), origin of replication (ori), and lacZ gene (containing the polylinker cloning site) are shown. Also shown are representative restriction sites within the anthrax fragment.

Figure 8. The scheme B format was used to generate standard curves for the anthrax target (gel purified) with both the single and dual fluorescein probes. Note that although the specific signals are comparable for both probes, the background signal for the dual fluorescein probe is higher (~500 uv/sec).

Figure 9. A. PCR reactions (50 µl) contained 200 µM dNTPs, 0.5 µM of each primer, 10^7 targets (gel purified 622 bp fragment), and 2.5 units of Pfu DNA polymerase (Stratagene; buffer supplied by manufacturer). Fluorescein-11-dUTP (1mM solution) was purchased from Amersham. Cycling parameters were as follows: 25 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 2 min and 1 cycle of 72°C, 7 min. 10 µl of each reaction was analyzed on 1.2% Seakem GTG agarose (FMC BioProducts) in 1X Tris Acetate EDTA buffer (25X stock purchased from AMRESCO). **B.** The scheme A/B format was used to generate standard curves using the single fluorescein probe and 10 µl of the FI-PCR reaction shown in lane 3 above. pKHant digested with *Bam* HI and *Sph* I was used as the target.

Figure 10. A. PCR reactions (50 µl) contained 200 µM dNTPs, 0.5 µM of primers (BACA1F1 and BACA6R1), 5 units of Taq polymerase (Promega; buffer supplied by manufacturer). The target was 0, 10, 100, 1000, or 10,000 copies of the *Bacillus anthracis* genome (supplied by Suresh Pillai at NMRI). FI-PCR reactions contained 100 µM dTTP and 100 µM FI-dUTP. Cycling conditions were as follows: 1 cycle of 94°C 5 min; 25 cycles of 94°C 1 min, 55°C 1 min, 72°C 2 min; 1 cycle of 72°C 7 min. 10 µl aliquots of each reaction were analyzed on 1.2% agarose in 1X TAE buffer. **B.** The scheme B format was used to assay 10 µl aliquots of each PCR reaction. Both biotin and fluorescein probes were added to the control reactions while only the biotin probe was added to assay the

reactions containing Fl-dUTP.

Figure 11. A. PCR reactions were set up with 10^4 *Bacillus anthracis* genomic targets and varying ratios of Fl-dUTP to dTTP. Reactions were cycled and analyzed as described in the legend to figure 11A. **B.** 5 µl aliquots of each reaction were assayed using the scheme B format using only the biotin probe. The positive control (dTTP only) and negative control (no target) PCR reactions were assayed with both the fluorescein and biotin probes.

Figure 1

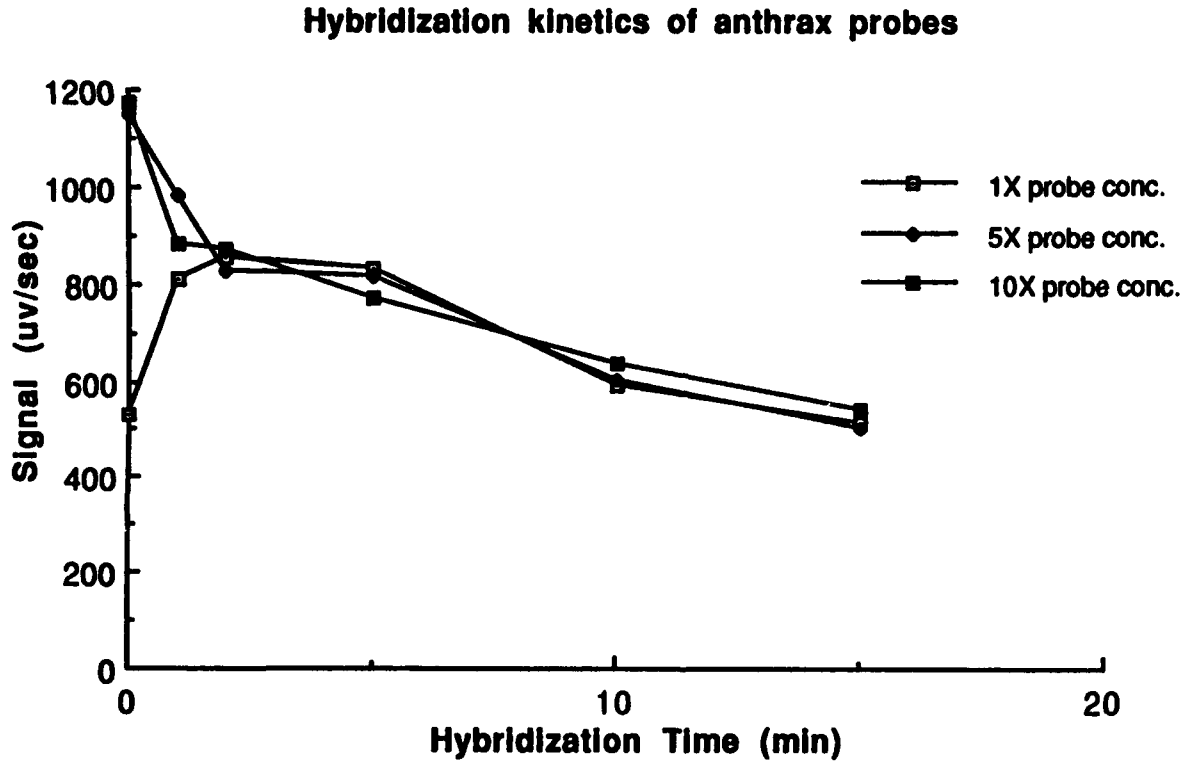
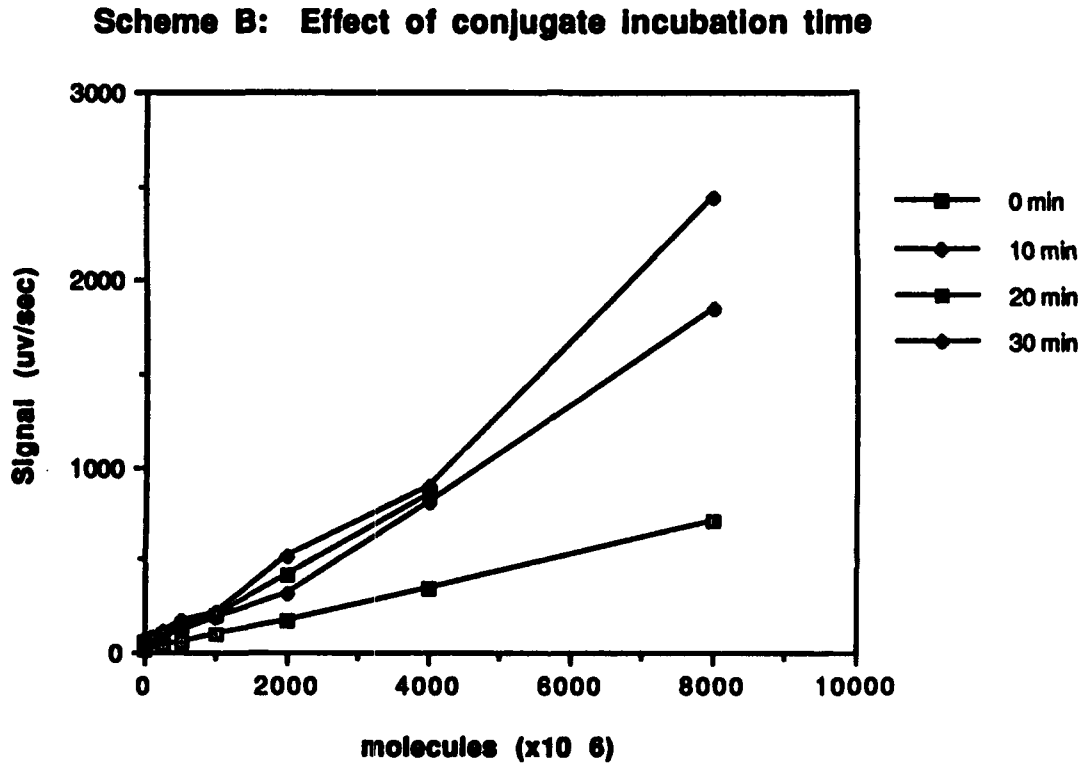

Hybridization kinetics of anthrax probes
Signal (uv/sec)
1200
1000
800
600
400
200
0
0
10
20
Hybridization Time (min)
1X probe conc.
5X probe conc.
10X probe conc.

Figure 2


Scheme B: Effect of conjugate incubation time
3000
2000
1000
0
Signal (uv/sec)
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2000
4000
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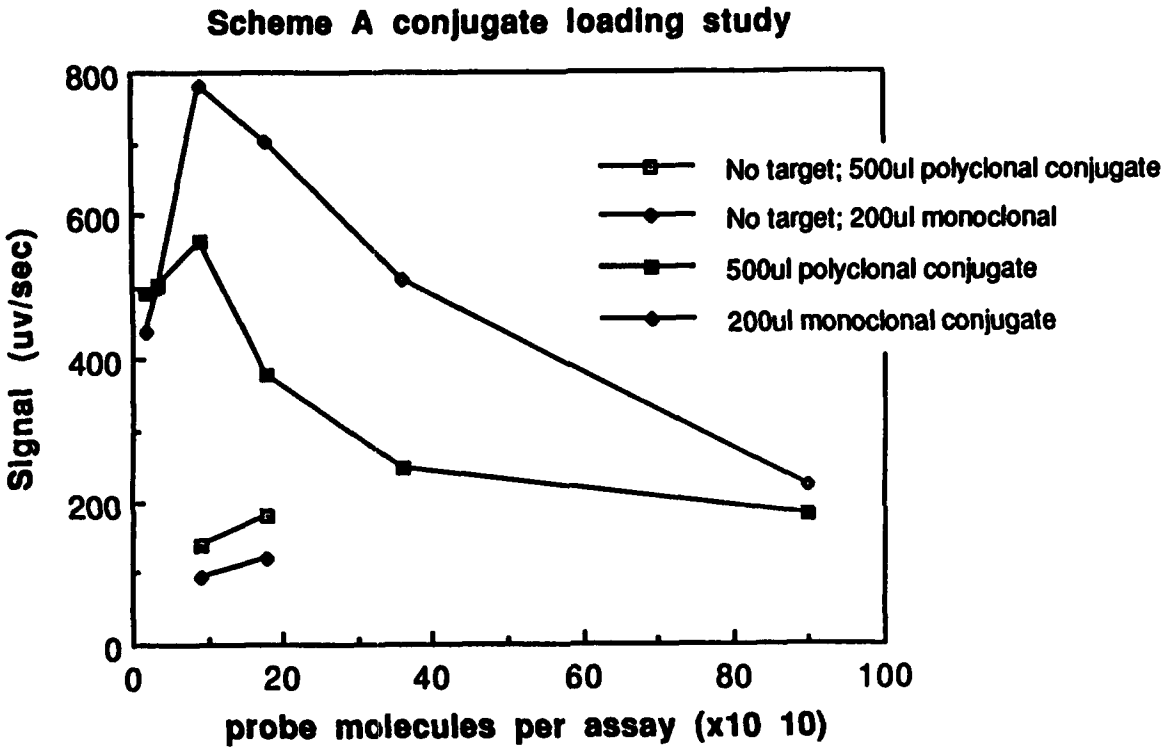
8000
10000
molecules (x10 6)
0 min
10 min
20 min
30 min

Figure 3


Scheme A conjugate loading study
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600
400
200
0
Signal (uv/sec)
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20
40
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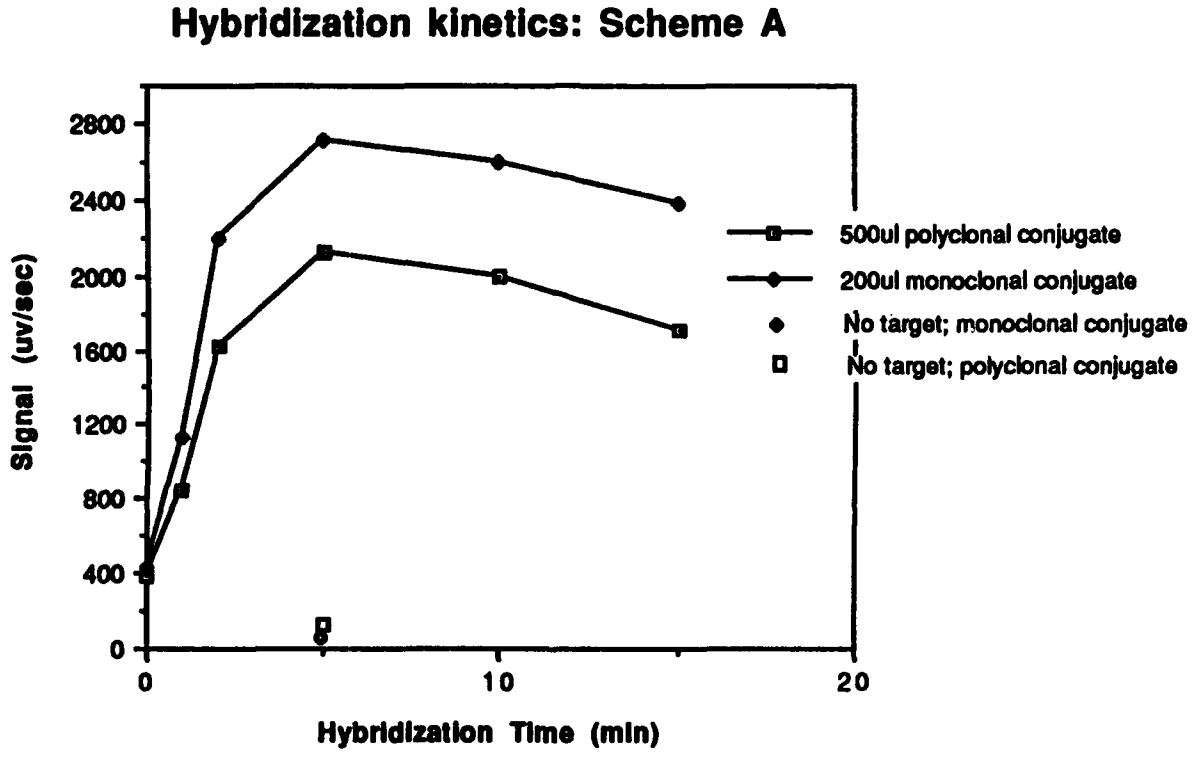
80
100
probe molecules per assay (x10 10)
No target; 500ul polyclonal conjugate
No target; 200ul monoclonal
500ul polyclonal conjugate
200ul monoclonal conjugate

Figure 4


Hybridization kinetics: Scheme A
Signal (uv/sec)
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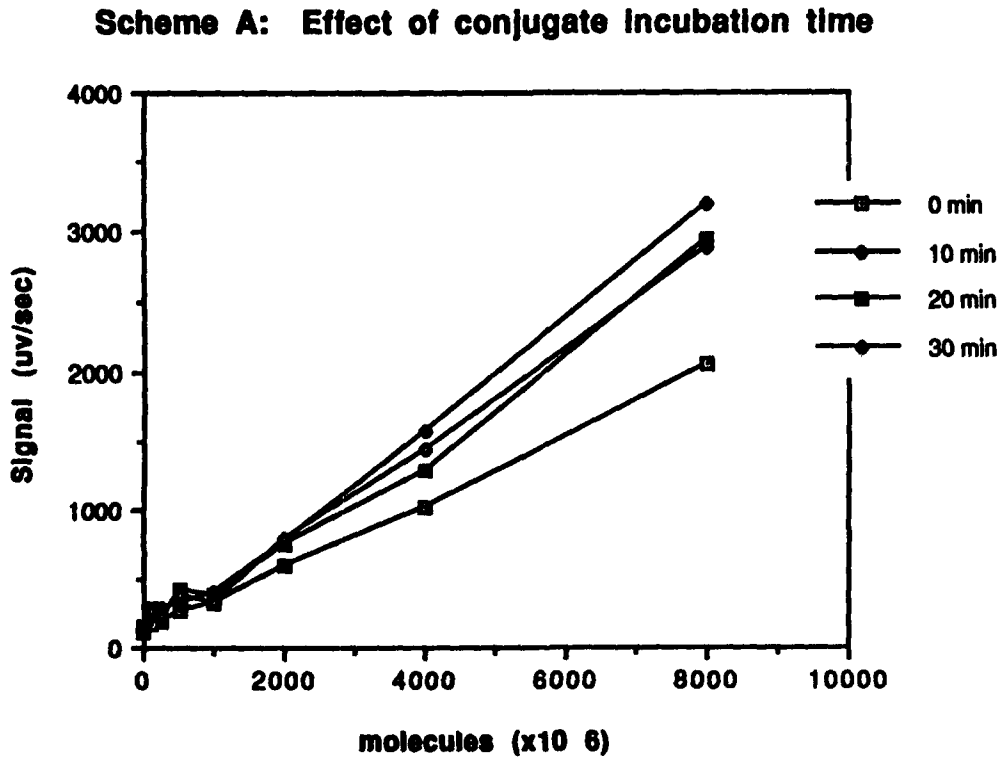
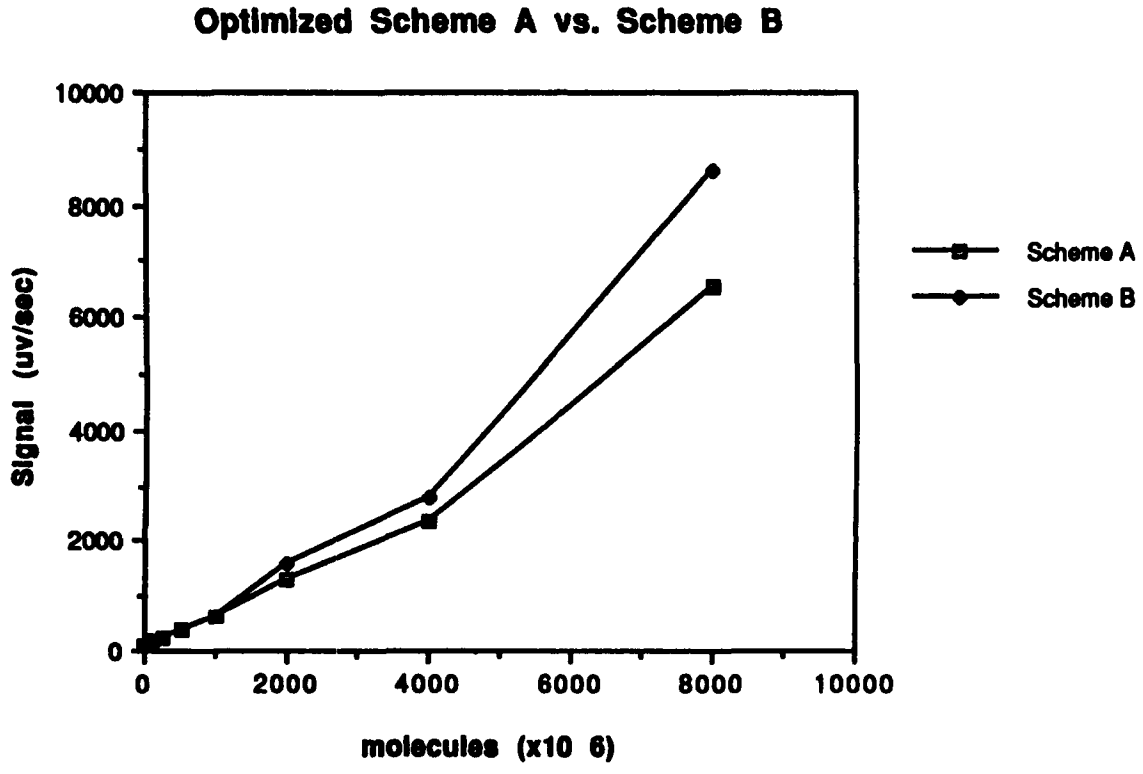
2400
2000
1600
1200
800
400
0
0
10
20
Hybridization Time (min)
500ul polyclonal conjugate
200ul monoclonal conjugate
No target; monoclonal conjugate
No target; polyclonal conjugate

Figure 5


Scheme A: Effect of conjugate incubation time
4000
3000
2000
1000
0
Signal (uv/sec)
0
2000
4000
6000
8000
10000
molecules (x10 6)
0 min
10 min
20 min
30 min


Optimized Scheme A vs. Scheme B
10000
8000
6000
4000
2000
0
Signal (uv/sec)
0
2000
4000
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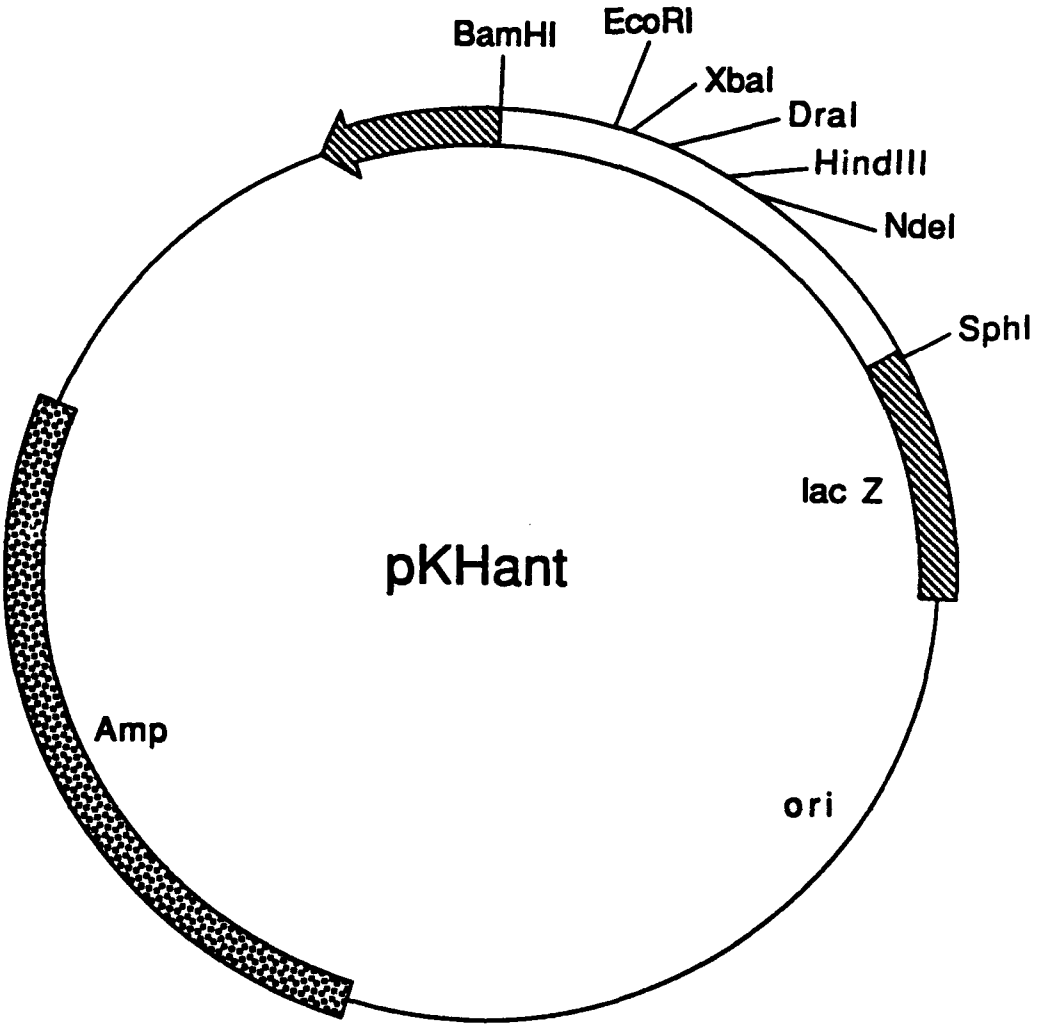
8000
10000
molecules (x10 6)
Scheme A
Scheme B

Figure 7



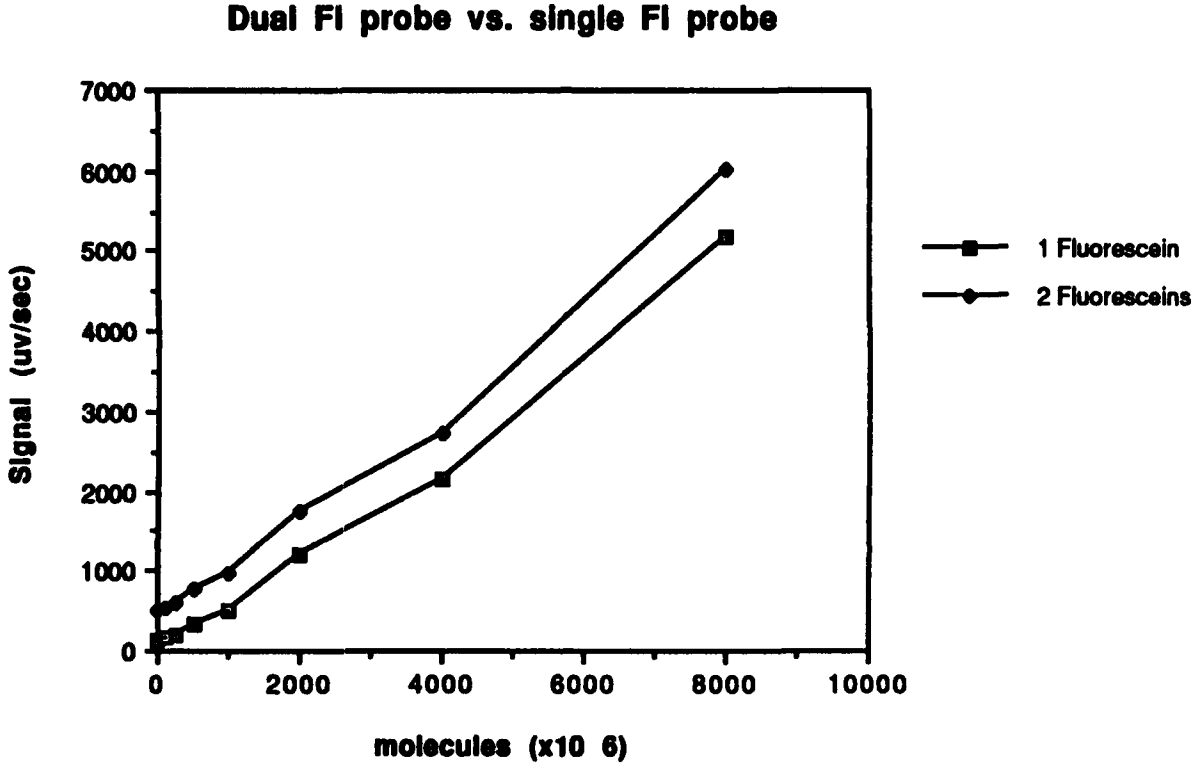
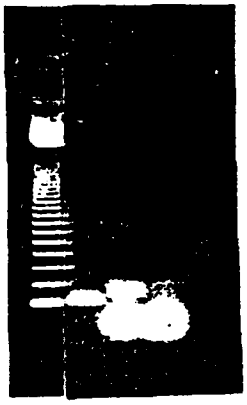

Dual Fl probe vs. single Fl probe
7000
6000
5000
4000
3000
2000
1000
0
Signal (uv/sec)
0
2000
4000
6000
8000
10000
molecules (x10 6)
1 Fluorescein
2 Fluoresceins

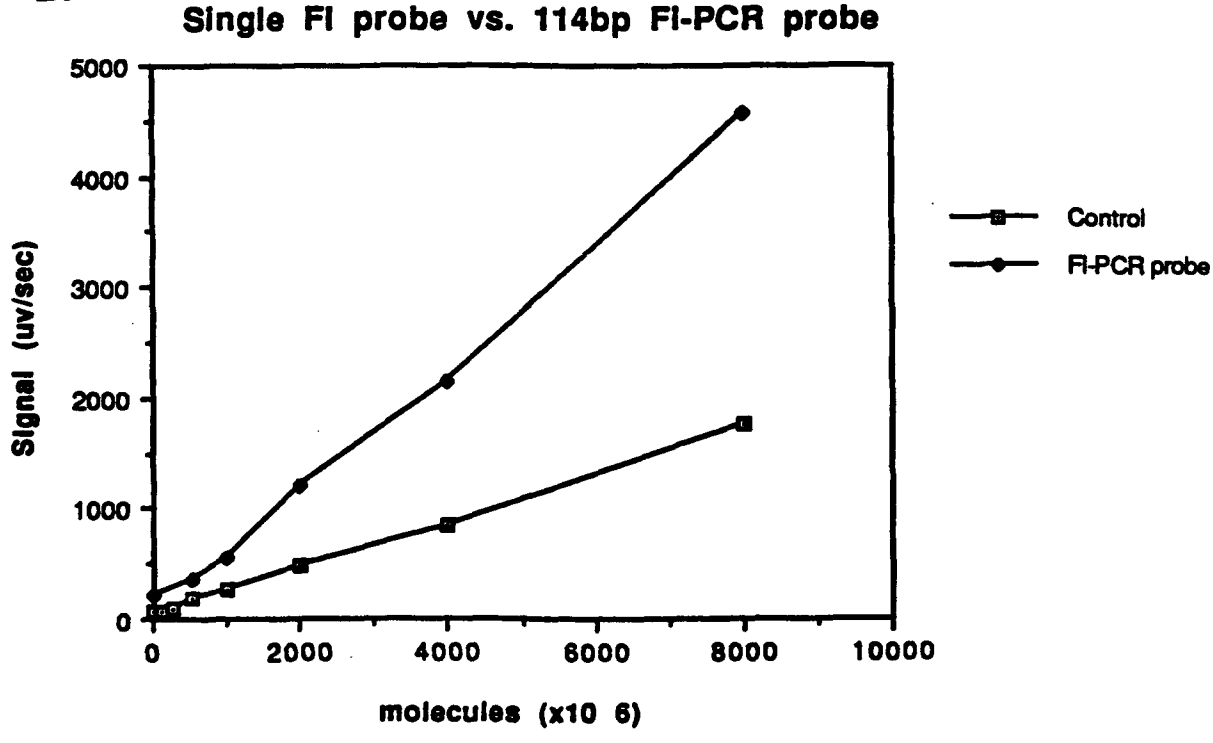
Figure 9

A.



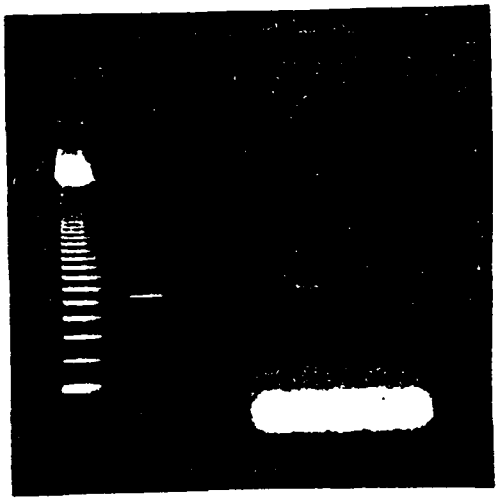
Lane	Sample
1	123 bp ladder
2	Control (100% dTTP)
3	75%Fl-dUTP:25%dTTP
4	100% Fl-dUTP

B.



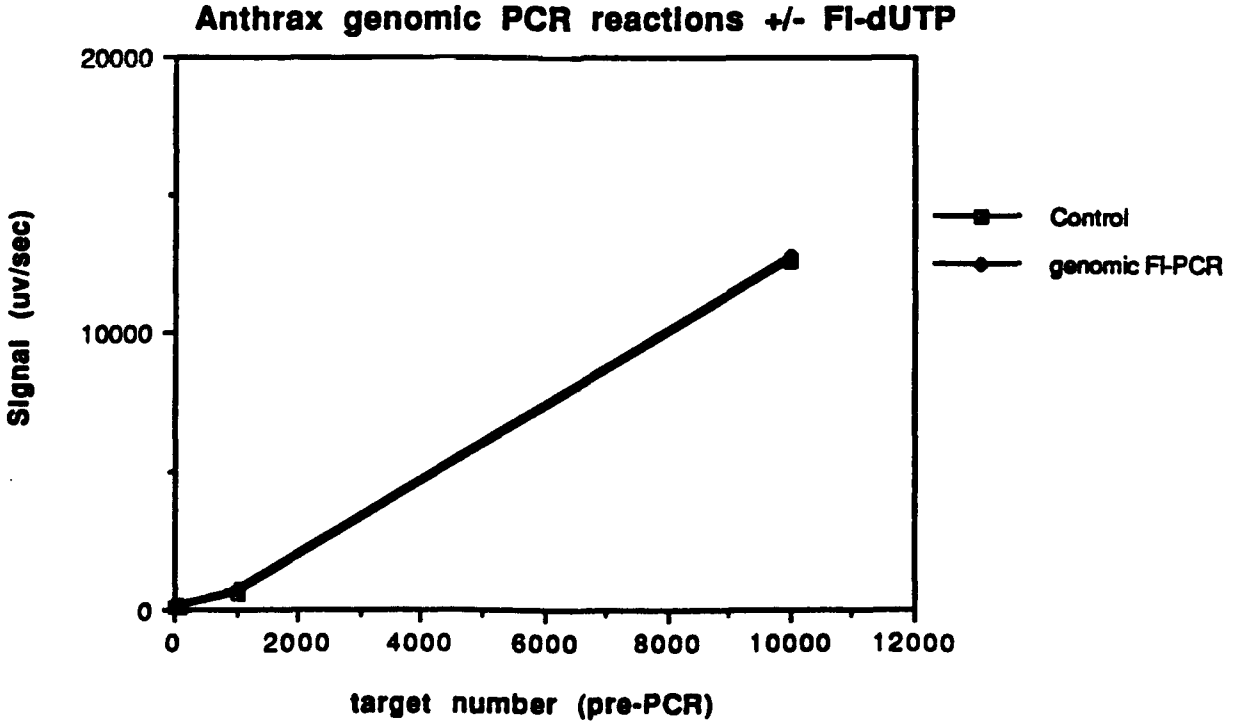
jure 10

A.



Lane	Sample
1	123 bp ladder
2	No target
3	10^4 targets
4	10^3 targets
5	10^2 targets
6	10^1 targets
7	No targets + Fl-dUTP
8	10^4 targets + Fl-dUTP
9	10^3 targets + Fl-dUTP
10	10^2 targets + Fl-dUTP
11	10^1 targets + Fl-dUTP

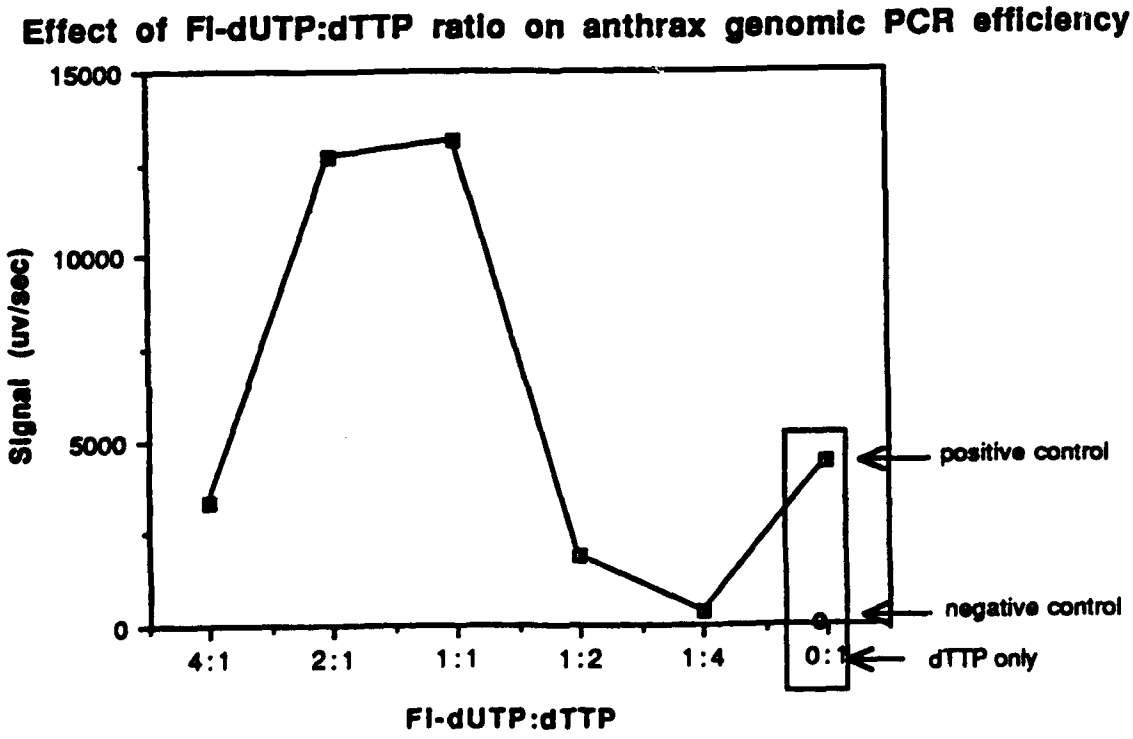
B.



A.

Lane	Sample
1	123 bp ladder
2	No target
3	Control: 200 µM dTTP
4	80%Fl-dUTP:20%dTTP
5	67%Fl-dUTP:33%dTTP
6	50%Fl-dUTP:50%dTTP
7	33%Fl-dUTP:67%dTTP
8	20%Fl-dUTP:80%dTTP

B.



APPENDIX I

SCHEME B DNA PROBE ASSAY

1.	Denature target at 100°C	5 min
2.	Anneal probes ($9x10^{11}$ molecules of each probe) at 53°C	0 min*
3.	Add 100µl of 10X capture reagent, filter, and wash	~10 min
4.	Incubate with 200µl αFl-urease conjugate (ILA)	30 min
5.	Filter and wash	~5 min
6.	Read sticks	~5 min

* In the optimized scheme B protocol the reactions are transferred to ice directly from the denaturation temperature and then immediately processed.

APPENDIX II

OPTIMIZED SCHEME A PROBE ASSAY

1.	Denature target at 100°C	5 min
2.	Anneal probes ($9x10^{10}$ molecules of each) at 53°C	5 min
3.	Add 100µl of 10X capture reagent and 200µl of αFl-urease conjugate (monoclonal), filter, and wash	~10 min
4.	Read sticks	~5 min

APPENDIX III

SCHEME A/B DNA PROBE ASSAY

1.	Denature target at 100°C	5 min
2.	Anneal probes ($9x10^{10}$ molecules of biotin probe and 10 µl of Fl-PCR reaction) at 53°C	5 min
3.	Add 100µl of 10X capture reagent, filter, and wash	~10 min
4.	Incubate with 200µl αFl-urease conjugate (ILA)	30 min
5.	Filter and wash	~5 min
6.	Read sticks	~5 min